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Induction of Oxidative Stress in Brain Tissues of Mice after Subchronic Exposure to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

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The ability of single doses of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) to induce oxidative stress in hepatic and some extrahepatic tissues of animals is well documented. However, no previous study has examined the ability of TCDD to induce oxidative stress and tissue damage in brain *in vivo*. In this study the ability of TCDD to induce oxidative stress in brain tissues of mice was studied after subchronic exposures. Groups of female B6C3F1 mice were treated orally with TCDD (0, 0.45, 1.5, 15, and 150 ng/kg/day) for 13 weeks, 5 days/week. The animals were euthanized 3 days after the last treatment and brain tissues were collected. Biomarkers of oxidative stress including production of superoxide anion, lipid peroxidation, and DNA–single-strand breaks (SSB) were determined. TCDD treatment resulted in significant and dose-dependent increases in the production of superoxide anion as assessed by reduction of cytochrome *c*. Significant increases were also observed in lipid peroxidation and DNA–SSB in those tissues, as assessed by the presence of thiobarbituric acid-reactive substances and the alkaline elution technique, respectively. These results clearly indicate that subchronic exposure to low doses of TCDD can induce oxidative tissue damage in brain tissues which may at least in part play a role in the effects of TCDD on the central nervous system.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is often called the most toxic man-made chemical. Despite the wide range of effects of TCDD in various animal species, the role of the central nervous system in TCDD intoxication has received relatively little attention. Data concerning the direct effects of TCDD on the central nervous system are contradictory. While studies by Pohjanvirta *et al.* (1989) have demonstrated a typical reduction in feed intake after slow infusion of TCDD into the brain ventricles of Long-Evans and Han/Wistar rats, these findings could not be replicated in Sprague-Dawley rats (Stahl and Rozman, 1990). TCDD does not readily penetrate into the brain, and concentrations of TCDD are far lower in the brain than in the liver or adipose tissues after exposure (Pohjanvirta *et al.*, 1990; Weber *et al.*, 1993). However, sensitive biochemical effects, such as enzyme induction (Elovaara *et al.*, 1977; Hook *et al.*, 1975, Unkila *et al.*, 1993, 1995) and changes in the concentrations of selected neurotransmitters (Russell *et al.*, 1988; Tuomisto *et al.*, 1990; Pohjanvirta *et al.*, 1994), are seen in the brain after TCDD exposure.

Recent studies also demonstrate a high frequency of intercerebral asymmetry in the brains of heron hatchlings from areas environmentally contaminated with TCDD, and this effect was significantly correlated with the levels of TCDD in eggs taken from the same nest (Henshel *et al.*, 1995). There is also preliminary evidence that TCDD may cause injury to rat hippocampal neurons in culture, as indicated by an increase in intracellular calcium (Hanneman *et al.*, 1993).

Oxidative stress is considered an important mechanism in the toxicity of TCDD (Stohs, 1990; Stohs *et al.*, 1991). Previous studies have shown that administration of TCDD to laboratory animals induces production of reactive oxygen species (Alsharif *et al.*, 1994a,b; Bagchi and Stohs, 1993), lipid peroxidation (Stohs *et al.*, 1983; Mohammadpour *et al.*, 1988), DNA damage (Wahba *et al.*, 1988; Alsharif *et al.*, 1994b), and decreased membrane fluidity (Alsharif *et al.*, 1990) following acute, high-dose exposure. TCDD administration to rats also results in significant increases in the urinary excretion of the lipid metabolites, malondialdehyde, formaldehyde, acetaldehyde, and acetone (Bagchi *et al.*, 1993). In the current study we have shown that oxidative tissue damage can occur in the brains of mice following subchronic exposure to doses of TCDD as low as 0.45 ng/kg/day.

MATERIALS AND METHODS

Chemicals. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin was obtained from Ultra Scientific (Northkings Town, RI) and was >98% pure. All other chemicals

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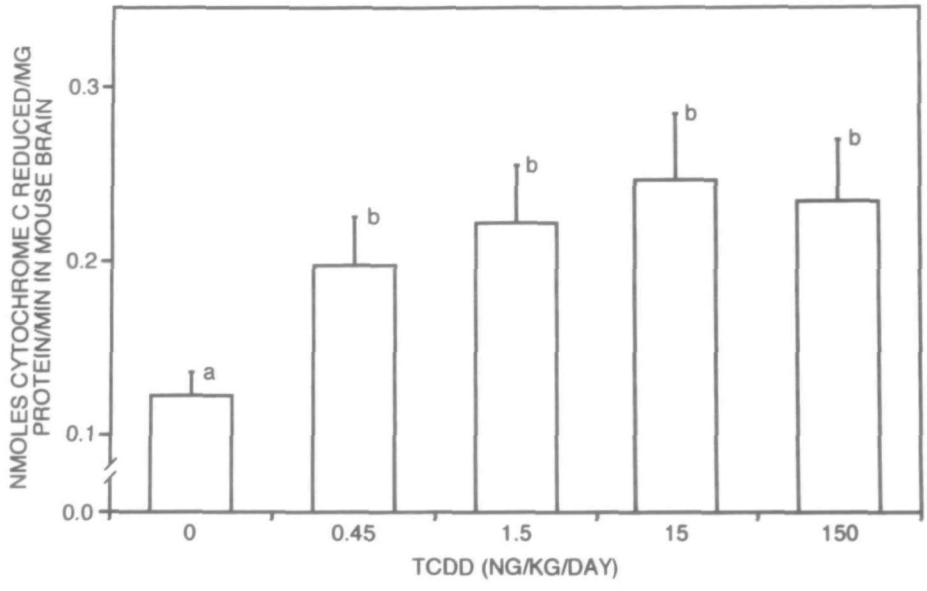

FIG. 1. Superoxide anion production by brain tissue homogenates based on cytochrome *c* reduction, expressed as nanomoles of cytochrome *c* reduced per milligram of protein per minute. Animals were treated with TCDD (treated groups) or the corn oil vehicle (control group) for 13 weeks (5 days/week) and were euthanized 3 days after the last dose. Values with nonidentical letters are significantly different ($p < 0.05$).

used in this study were obtained from Sigma Chemical Co. (St. Louis, MO) and were of analytical grade or of the highest grade commercially available.

Animals and treatments. Female B6C3F1 mice were obtained from Charles River (Raleigh, NC) at 60 days of age and were maintained and treated at the U.S. Environmental Protection Agency (EPA) (Research Triangle Park, NC). The animals were housed at 25°C with a 12-h light/dark cycle and were given a commercial pelleted diet with tap water *ad libitum*. TCDD solutions were administered to the different groups of mice by gavage at doses of 0.45, 1.5, 15, and 150 ng/kg/day for 13 weeks, 5 days/week (DeVito and Birnbaum, 1994). Control mice received the corn oil vehicle used to dissolve TCDD at 10 ml/kg/day. The animals were euthanized 3 days after the last treatment using carbon dioxide asphyxiation, and the brains were removed and maintained over dry ice until determination of oxidative tissue damage.

Sample preparation. Brain tissues from each mouse were divided into two portions, weighed, and placed either in Tris–KCl buffer (0.05 M Tris chloride and 1.15% KCl, pH 7.4) for lipid peroxidation and production of reactive oxygen species assays or in the homogenizing buffer of White *et al.* (1981) for the assay of DNA–single-strand breaks (DNA–SSB). The samples were homogenized over ice using a Potter-Elvehjem homogenizer fitted with a Teflon pestle to produce a 10% homogenate for lipid peroxidation and superoxide anion assays and a 20% homogenate for the assay of DNA–SSB. The DNA homogenates were centrifuged at 1000*g* for 10 min. After centrifugation, nuclear pellets were resuspended in two times the original volume of the homogenizing buffer for the determination of DNA–SSB.

Determination of lipid peroxidation. Lipid peroxidation was determined in brain tissues using the colorimetric method of Uchiyama and Mihara (1978) by measuring the formation of thiobarbituric acid-reactive substances (TBARS). Malondialdehyde was employed as the standard. The TBARS concentrations were determined spectrophotometrically on a Perkin-Elmer spectrophotometer, utilizing the differences at 535 and 520 nm and the molar absorptivity constant of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

Determination of the production of reactive oxygen species. Production of superoxide anion in brain tissue homogenates was measured by the assay method of Babior *et al.* (1973), which is based on the reduction of cytochrome *c* collected for subsequent spectrophotometric measurement. Absorbances were monitored at 550 nm and converted to nanomoles of cytochrome *c* reduced/min, using the extinction coefficient $2.1 \times 10^4\ M^{-1}\ cm^{-1}$.

Determination of DNA–single-strand breaks. DNA damage was measured in the nuclear fractions of brain tissues as single-strand breaks by the alkaline elution method as previously described (Wahba *et al.*, 1988) DNA contents were measured microfluorometrically (excitation 436 nm, emission 521 nm) in a Perkin-Elmer spectrofluorometer and the elution constant (k) was determined by plotting the $\log_{10}$ DNA remaining on the filter after each fraction, against the volume of the eluate, where $k = -2.3 \times$ slope of this plot.

Determination of protein. Protein concentrations were determined by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard.

Statistical methods. Data are expressed as the mean ± standard deviation (SD). Each value was derived from seven animals. Data for each group of treated animals were compared with the control values, using analysis of variance (ANOVA) with Scheffe's S method as the post hoc test. The level of statistical significance employed in all cases was $P < 0.05$.

RESULTS

The ability of various doses of TCDD to induce production of superoxide anion in brain tissues of B6C3F1 mice after subchronic exposure is presented in Fig. 1. Significant increases in superoxide anion production, as indicated by reductions of cytochrome *c*, were observed, relative to control levels. These increases were dose dependent, being first significantly detected at 0.45 ng/kg/day compared to controls, with a plateau being observed at a TCDD dose of 15 ng/kg/day. At this dose a 2.3-fold increase in cytochrome *c* reduction (Fig. 1) was observed.

The effect of subchronic, low-dose administration of TCDD to B6C3F1 mice on the induction of lipid peroxidation (TBARS formation) in brain tissues is presented in Fig. 2.

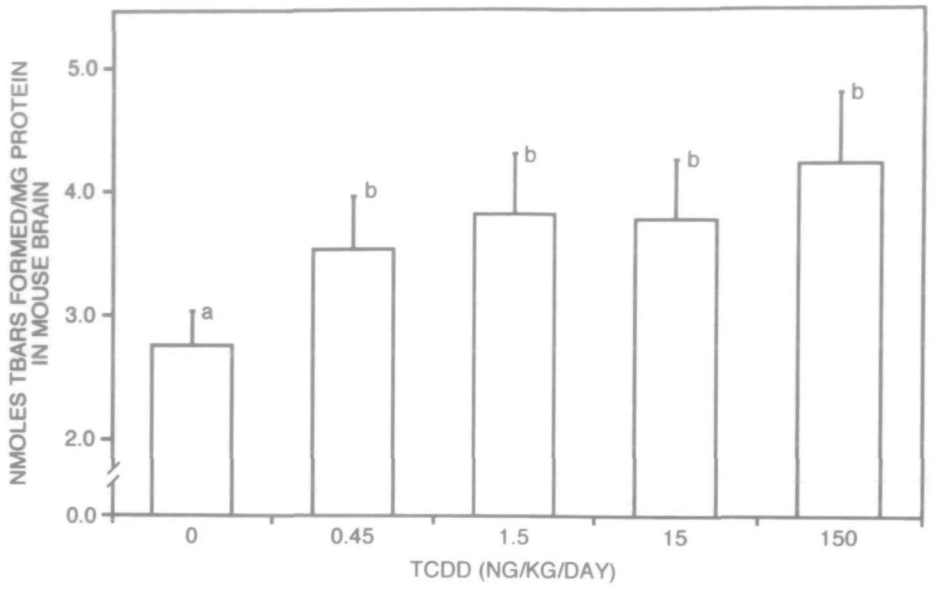

FIG. 2. Lipid peroxidation based on the formation of thiobarbituric acid reactive substances (TBARS), expressed as nanomoles of malondialdehyde (MDA) per milligram of protein. Animals were treated with TCDD (treated groups) or corn oil vehicle (control group) for 13 weeks (5 days/week) and were euthanized 3 days after the last dose. Values with nonidentical letters are significantly different ($p < 0.05$).

Increases in lipid peroxidation were observed at all four doses relative to the control group, with a 1.4-fold increase in TBARS formation occurring at a 0.45 ng/kg/day dose of TCDD and a 1.8-fold increase occurring at a dose of 150 ng/kg/day.

The effect of TCDD on DNA–SSB in brain tissues of B6C3F1 mice is presented in Fig. 3. A dose-dependent increase in DNA–SSB was observed relative to the control group. At a TCDD dose of 0.45 ng/kg/day a 2.0-fold increase in DNA–SSB was observed, while at 150 ng/kg/day a 3.5-fold increase in DNA–SSB occurred.

DISCUSSION

The present studies clearly demonstrate that subchronic exposure to TCDD induces oxidative stress in brain tissues of mice as evidenced by the enhanced production of superoxide anion (Fig. 1) and the increased production of tissue damage as suggested by the induction of lipid peroxidation (Fig. 2) and DNA damage (Fig. 3). It has been shown that the brain consumes a proportionally larger amount of oxygen than other organs and has only moderate levels of both enzymatic and nonenzymatic scavengers of reactive oxygen species (Halliwell and Gutteridge, 1985). Lipid peroxidation is one of the primary effects induced by oxidative stress and may occur readily in the brain due to the presence of membranes that are rich in polyunsaturated highly oxidizable fatty acids (Cini *et al.*, 1994). Thus, the brain may be a target organ for various chemicals such as TCDD which are known to induce oxidative tissue damage.

The results of the current study agree well with previous results demonstrating the ability of TCDD to induce production of reactive oxygen species as well as lipid peroxidation and DNA damage in hepatic and extrahepatic tissues other than brain (Stohs, 1990; Stohs *et al.*, 1991; Mohammadpour *et al.*, 1988; Bagchi *et al.*, 1994; Alsharif *et al.*, 1990, 1994a,b; Wahba *et al.*, 1988). Oxidative stress has been demonstrated in embryonic tissues of TCDD-responsive mice and may be due to the extremely low concentrations of TCDD which are transferred to the embryos after maternal exposure to a low dose of the xenobiotic (Hassoun and Stohs, 1996; Hassoun *et al.*, 1995).

Previous studies have involved single doses of TCDD ranging from 5 to 125 μg/kg in TCDD-responsive animals (Mohammadpour *et al.*, 1988; Stohs *et al.*, 1991; Alsharif *et al.*, 1990; Wahba *et al.*, 1988). The total doses received in the current study ranged from 29.25 to 9750 ng/kg. The two highest doses used in the current study (15 and 150 ng/kg/day) cumulatively are in the range of doses previously used on a single dose basis. However, the two lowest doses which were used in the current study (0.45 and 1.5 ng/kg/day) are well below doses previously used to assess the ability of TCDD to induce oxidative stress and lipid peroxidation. Thus, the current results demonstrate that a dose as low as 0.45 ng/kg/day of TCDD given on a subchronic basis (13 weeks) can result in significant increases in production of reactive oxygen species as well as enhanced lipid peroxidation and DNA damage in brain tissues of mice. In mice receiving 1.5 ng/kg/day for 13 weeks, brain concentrations of TCDD were approximately 3.0 pg/g of tissue (unpublished observations), indicating that TCDD accumulates in brain tissues and measurable quantities can be

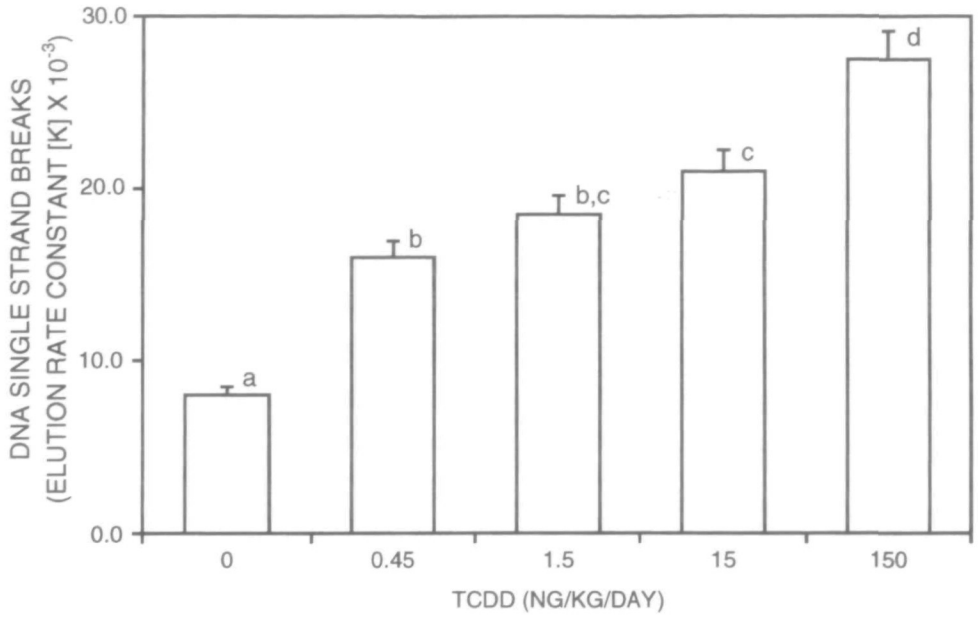

FIG. 3. DNA–single-strand breaks (SSB) were determined by the alkaline elution method and are presented as the elution rate constants (k). Animals were treated with TCDD (treated group) or the corn oil vehicle (control group) for 13 weeks (5 days/week) and were euthanized 3 days after the last treatment. Values with nonidentical letters are significantly different ($p < 0.05$).

detected. Average human body burdens of TCDD in the United States are estimated at 5–10 ng TEQ/kg (De Vito *et al.*, 1995).

Various possible mechanisms may be involved in the TCDD-induced production of reactive oxygen species in brain tissues. One of these mechanisms may involve the cytochrome P450 system. The ability of this system to produce reactive oxygen species has been well documented (Bondy and Naderi, 1994). Cytochrome P450 is induced in brain tissues in response to TCDD (Unkila *et al.*, 1995) and may therefore account for the formation of reactive oxygen species. However, additional studies will be required to correlate cytochrome P450 with the effects observed in the present study.

Another possible mechanism in the production of reactive oxygen species by TCDD may involve the induction of aldehyde dehydrogenase activity. The ability of this enzyme to generate oxygen free radicals as products of its catalytic cycle has been documented (Martinez-Cayuela, 1995). Dose-dependent increases in the activity of aldehyde dehydrogenase in brain tissue of rats are observed following *in vivo* treatment with TCDD at doses ranging from 5 to 50 μg/kg body wt (Unkila *et al.*, 1993). This induction may contribute to overproduction of reactive oxygen species in brain tissues in response to TCDD administration. In addition, TCDD enhances production of reactive oxygen species as superoxide anion by mitochondria (Wahba *et al.*, 1988; Stohs *et al.*, 1991) and macrophages (Alsharif *et al.*, 1994). Thus, multiple sites and mechanisms may be involved in the production of reactive oxygen species in response to TCDD administration.

In summary, subchronic exposure to TCDD induces production of reactive oxygen species which results in the induction of oxidative damage in brain tissues of mice as evidenced by lipid peroxidation and DNA damage. Further investigations will be required to assess the role of this mechanism in TCDD-induced biochemical changes in the central nervous system as well as functional and morphological alterations which may be produced.

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